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THROMBIN-INHIBITORY ACTIVITY OF AQUEOUS LEAF AND FLOWER EXTRACT OF CATHARANTHUS ROSEUS

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ABSTRACT

Objective: To investigate the thrombin inhibitory activity of aqueous extracts of leaf and flower of *Catharanthus roseus*.
Methods: Thrombin-inhibitory activity was evaluated by thrombin inhibition assay *in vitro* and thrombin generation assay on rat plasma.

Results: We observed that both crude extract of leaf and flower of *C. roseus* inhibited thrombin activity and thrombin generation significantly from that of vehicle control and heparin. Further, we evaluated the thrombin inhibitory activity of Vinblastin sulfate and found that Vinblastin sulfate inhibits thrombin activity suggesting that thrombin inhibitory activity of crude extract of leaf and flower of *C.roseus* may be through Vinblastin sulfate.

Conclusions: The active principles of crude extract of leaf and flower of *C. roseus* may further help in the treatment of cancer-associated thrombosis.

KEYWORDS: *Catharanthus roseus*, Thrombin, Thrombosis, Vinblastin

INTRODUCTION

Cardiovascular disease is one of the leading causes of morbidity and mortality worldwide (Murray and Lopez, 1997). Intravascular thrombosis is the major events in cardiovascular diseases. There is increased activation of coagulation factors resulting in thrombin formation in pathologies like diabetes, atherosclerosis, deep vein thrombosis (DVT) and disseminated intravascular coagulation (DIC) (Tripodi *et al*., 2011; Von Kaulla *et al*., 1966; Kitchens, 2009). Further, it leads to formation of intravascular thrombosis, occluding blood vessels and resulting death.

There are many drugs available in the market to inhibit the intravascular thrombosis (Melnikova, 2009). However, there is increased bleeding diathesis, recurrent stenosis and thrombocytopenia associated with these medications (Datta *et al*., 2011; Windecker and Meier, 2000). Deficiency of many coagulation factors like Protein C and Protein S can lead to deep vein thrombosis (Modal *et al*., 2010). Warfarin is one of the widely used oral anticoagulant however; it is associated with drug interactions, slow onset of actions and variable response (Datta *et al*., 2011). Therefore, there is a quest to develop newer compounds to inhibit intravascular thrombosis (Sikka and Bindra, 2010).

Natural products have been the source of various remedies. Therefore, we collected the leaves and flow from a plant named *Catharanthus roseus* and evaluated its effect on thrombin. *Catharanthus roseus* (*Vinca rosea*) is a plant belongs to the family *Apocynanceae* of the order *Gentianales* under class *Magnaoliposida dicotylendos*. Various medicinal properties have been ascribed to this plant and plant parts. It has antifungal (Roy and Chatterjee, 2010), anti-bacterial (Patil and Ghosh, 2010), anti-oxidant (Ferreres *et al*., 2008) and antihyperglycemic (Singh *et al*., 2001) properties. Many

useful alkaloids have been isolated from this plant like vindesine, vincristine and vinblastin, which have been used for treatment of chronic myelocytic leukemia (Baccarani *et al*., 1982). Therefore, we investigated the effect of *Catharanthus roseus* extract for its effect against thrombin.

MATERIALS AND METHODS

Materials

Catharanthus roseus leaves and flowers were obtained locally. Tris base, Thrombin, Heparin and Vinblastin sulfate were from Sigma Chemicals Inc. (USA) and Thrombin Substrate III from Calbiochem (USA). All the other chemicals used were of analytical grade.

Animals

In vitro thrombin generation experiments were performed on male wistar rats (250–300 g). All the animals were kept in polypropylene cages and maintained at 24 ± 0.5 °C, 12 h day/night cycle in the Central Animal Facility of the Indian Institute of Science. They were provided with chow pellets and water *ad libitum*. All the experiments were performed in accordance with the ethical and animal care guidelines of the Institute.

Extraction Procedure

The aqueous extraction method followed was as described by Ramya et al., 2008. Briefly, the *Catharanthus roseus* leaves were collected and dried at 37 °C for 48 hours in an incubator. The leaves were ground in a grinding machine and exposure to direct sunlight was avoided to prevent the loss of active component. 50g of selected fresh leaf material were macerated with 50ml of distilled water in a grinding machine for about 10-15 minutes.

The macerated was filtered with double layer muslin cloth. The filtrate was centrifuged at 3500rpm for 30 minutes. The supernatant was filtrated through the Whatman No: 1 filter paper and stored at 5 °C (Ramya *et al*., 2008).

Thrombin Inhibition Assay

The thrombin inhibition assay was conducted as described by Batra et al., 2004. Briefly, the aqueous crude leaf or flower extract (5-100µg/ml) was incubated with Tris-buffer, pH 7.5 in a black 96-well plate. Then thrombin substrate III was added (0.2mM) followed by addition of thrombin (1U/ml). 96-well plate was read at 450nm of emission and 390nm of excitation in a fluorimeter (SpectraMax M5e, Molecular Devices, Inc., USA) (Batra *et al*., 2004).

Thrombin Generation Assay

The thrombin generation assay was conducted as described by Hemker et al., 1993 with slight modifications. Briefly, the blood was collected by cardiac puncture of the ether anaesthetized male wistar rats into a syringe containing 3.8% trisodium citrate (9:1, v/v). It was centrifuged at 2000rpm for 20 min at 20 ºC and plasma was obtained.

The aqueous crude leaf or flower extract (5-100µg/ml) was incubated with plasma obtained from rat in a 96 well plate (black, flat bottom). Then thrombin substrate III was added (0.2mM) followed by addition of thrombin (0.1U/ml). Fluorescence was read at 450nm of emission and 390nm of excitation in a fluorimeter SpectraMax M5e, Molecular Devices, Inc., USA (Batra *et al*., 2004; Debaugnies *et al*., 2010).

Statistical Analysis

Values have been reported as the Mean±SE in all the groups. Comparisons between the different groups were performed by student's t-test and differences were considered significant at $p<0.05$.

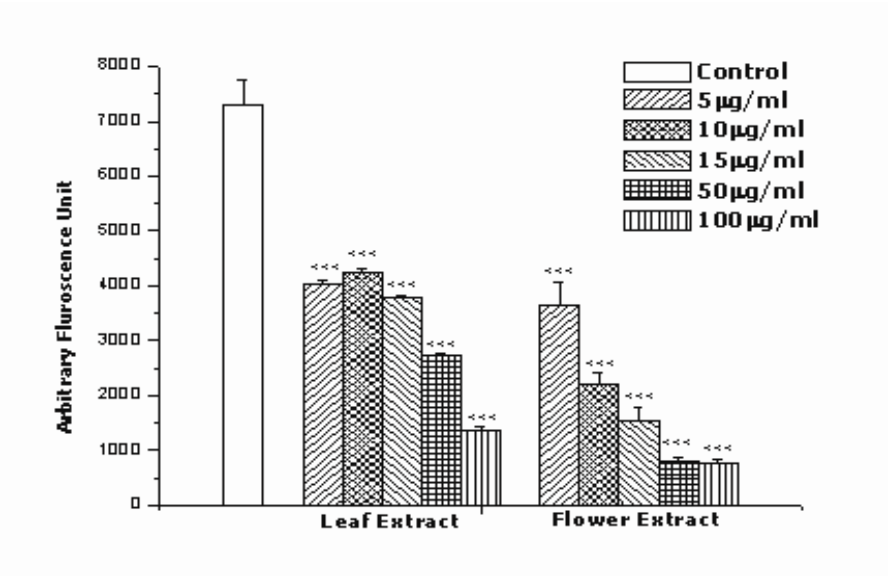

Figure 1: Thrombin Inhibitory Effect of *C. roseus* Aqueous Crude Extracts *in vitro* (for ***: p<0.001)

RESULTS

Effect of Aqueous Crude Extracts on Thrombin Activity

The crude extracts were incubated with the thrombin substrate at 5-100µg/ml concentrations for 5 min followed by addition of thrombin (1U/ml). The flouroscence was read out was taken by fluoroscence reader after 90 minutes. The arbitrary fluorescence unit (AFU) obtained in control was 7315±432.

With the treatment of AcqLE extract there was 41%, 49%, 57%, 77% and 82% inhibition of thrombin activity at 5µg/ml, 10µg/ml, 15µg/ml, 50µg/ml and 100µg/ml respectively. Whereas, with the treatment of AcqFlE extract there was 82%, 88%, 90%, 94% and 95% inihibition of thrombin activity at 5µg/ml, 10µg/ml, 15µg/ml, 50µg/ml and 100µg/ml concentrations respectively (Figure 1). This suggests that both the extracts can exert thrombin inhibitory effect in a concentration dependent manner.

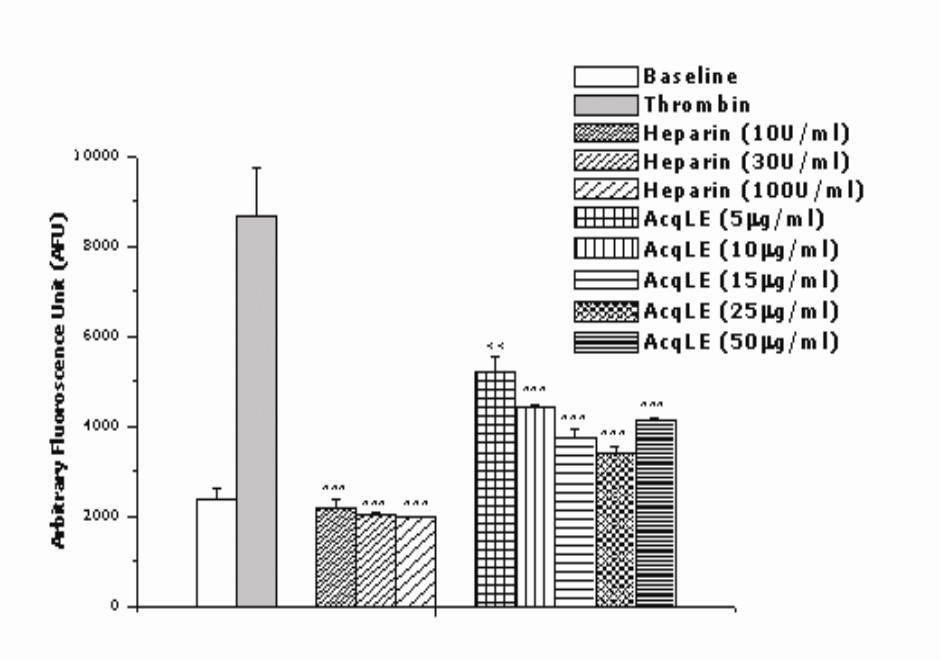

Figure 2: Effect of *C. roseus* Aqueous Crude Leaf Extract on Thrombin Generation in Rat Plasma (for **: p<0.01, for ***: p<0.001)

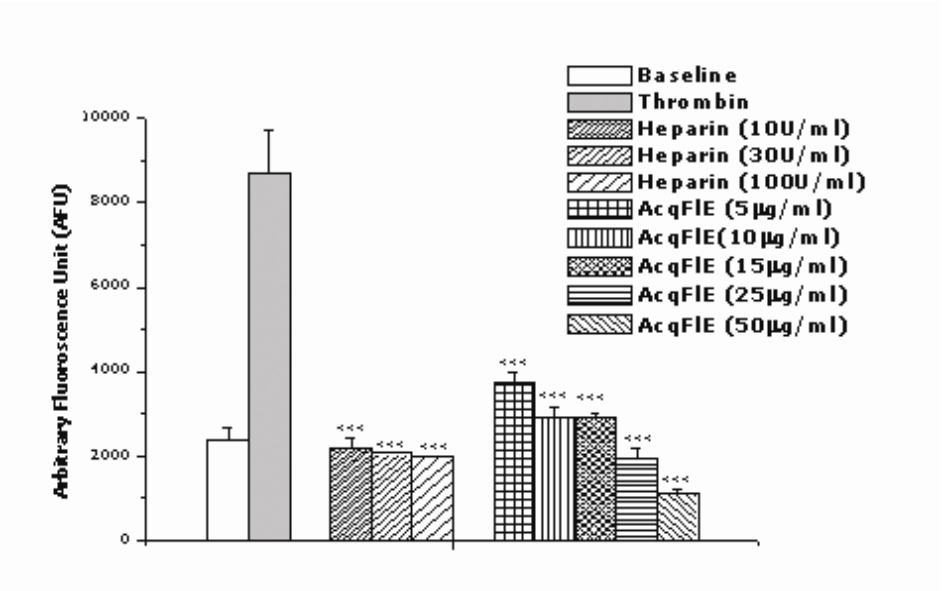

Figure 3: Effect of *C. roseus* Aqueous Crude Flower Extracts on Thrombin Generation in Rat Plasma (for ***: p<0.001)

Effect of Aqueous Crude Extracts on Thrombin Generation in Rat Plasma

Further, we evaluated the effect of the extracts againts thrombin generation. Endogenous thrombin potential determines the overall thrombin generation potential of the plasma. Moreover, there are no thrombin inhibitors available which can exert thrombin inhibition *in vitro;* we took rat plasma and evaluated the thrombin inhibitory activity of the crude extracts against thrombin generation in.

We took heparin as a standard compound for comparision of the thrombin generation inhibitory activity of the crude extracts. Anti-coagulant compounds generally bring about anti-coagulant effect by overall reduction in the endogenous thrombin generation potentatial of the plasma.

The acques crude extract inhibited thrombin generation in a concentration dependent manner. The AFU obtained in control group was 8697±1031. With treatment of heparin, there was 74%, 76% and 77% reduction of thrombin generation at 10U/ml, 30U/ml and 100U/ml of heparin respectively. Aqueous crude leaf extract (AcqLE) exhibited reduction in thrombin generation activity.

There was 40%, 40%, 57%, 60% and 53% reduction in thrombin generation with the treatment of AcqLE at 5µg/ml, 10µg/ml, 15µg/ml, 25µg/ml and 50µg/ml respectively (Figure 2). Further, the inhibition observed with the treatment of AcqFlE at 5µg/ml, 10µg/ml, 15µg/ml, 25µg/ml or 50µg/ml was 57%, 73%, 66%, 77% and 87% respectively suggesting that flower extract also inhibits the thrombin generation in a concentration dependent manner (Figure 3).

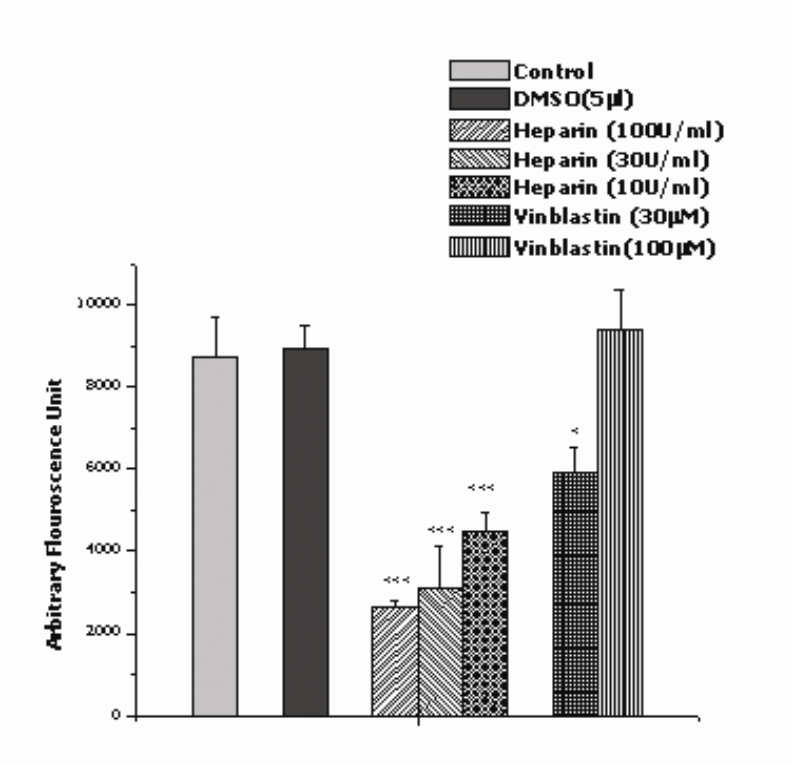

Figure 4: Effect of Vinblastin Sulfate and Heparin on Thrombin Generation in Rat Plasma *in vitro* (for **: p<0.05, for ***: p<0.001)

Effect of Vinblastin Sulfate and Heparin on Thrombin Generation in Rat Plasma

Vinblastin sulfate and vincristine sulphate are the active constituent of the *C. roseus* crude extracts. Therefore, we interested to evaluate if any of the active constituents possess thrombin inhibitory activity. Hence, we evaluated vinblastin sulfate for its effect on thrombin generation on rat plasma *in vitro*. We found that Vinblastin sulfate posees significant inhibitory effect against thrombin generation in rat plasma at 100µM concentration (Figure 3). This suggests that the thrombin inhibition by *Catharanthus roseus* crude extracts seems due to the presence of Vinblastin sulfate.

DISCUSSIONS

Natural products have been main source of antithrombotic compounds like aspirin, warfarin, and heparin (Wang and Ng, 1999). Various natural products possess anti-thrombotic effects (Sharma and Arora, 2005). Herbal therapies have gained attention as they exhibit fewer side effects. Better understanding of the disease process and advancement of modern techniques have helped in purification and characterization of active principles from the natural sources (Prakash *et al*., 2011; Surin and Dikshit, 2006). Aspirin was developed from a secondary metabolite produced from the willow leaves. McLean discovered heparin accidentally (Best, 1959). Streptokinase and urokinase, which dissolves clots, are naturally derived products (Surin and Dikshit, 2006; Wang and Ng, 1999).

There are various approaches to study the anti-thrombotic activity of the test compounds, like platelet aggregation, coagulation parameters. Present trend is to see the over all activity of test compounds to endogenous thrombin generation potential of the test compounds (Tripodi *et al*., 2007). Moreover, it simulates *in vivo* conditions and more relevant than coagulation parameters studies where specific pathways are investigated and various factors responsible for the coagulation inhibition are looked for (Tripodi *et al*., 2007).

In the present study we observed that *C. roseus* extracts inhibits thrombin activity. The thrombin inhibitory activity of *C. roseus* extracts has also been reported earlier (Ah Kee *et al*., 2008). However, its effect on endogenous

thrombin generation potential has not been studied. Thrombin generation of the plasma varies in different conditions (Tripodi *et al*., 2011; Von Kaulla *et al*., 1966). The thrombin generation potential of hypercoagulable blood in various pathologies and complications, which often leads to deep vein thrombosis, is much higher than blood obtained from a normal person (Butenas *et al*., 1999; van Hylckama Vlieg *et al*., 2007). Many anti-thrombotic compounds inhibit the activity of various coagulation factors and thereby reduce the thrombin generation potential (Hemker *et al*., 2004). However, many anti-thrombotic molecules do not inhibit the thrombin generation potential and it may lead to prothrombotic conditions (Monroe *et al*., 2002; Hemker *et al*., 2004).

Therefore, compounds that can inhibit endogenous thrombin generation in the plasma are highly beneficial for anti-coagulant therapies. Many compounds, which have inhibitory effect on coagulation factors, other than thrombin, may not exhibit thrombin inhibition activity *in vitro*. Thrombin generation simulate the *in vivo* conditions of the blood and plasma (Castoldi and Rosing, 2011). Moreover, we observed that one of the constituents of *C. roseus* extract Viblastin sulfate could reduce thrombin generation. This confirms that *Catharanthus roseus* extracts has thrombin inhibitory activity. Further, studies are required to delineate the mechanism of anti-thrombotic activity of vinblastin sulfate. Many active principles isolated from *C. roseus* exhibits anti- cancer effect (Baccarani *et al*., 1982).

These are effective against chronic myeloid leukemia. It has been observed that there is increased generation of thrombin and coagulation factors during cancer (Green and Karpatkin, 2010; Ay and Pabinger, 2010). Thrombin, the key initiator of intravascular thrombosis has been implicated in cancer-associated thrombosis (Jenkins *et al*., 2010). There are quest for antithrombotic drugs, which can exhibit beneficial effect during cancer malignancies (Lee, 2010; Coleman and MacCallum, 2010). These observations will certainly help in addressing cancer-associated thrombosis by novel therapies.

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Conflicts of Interest

Authors declare no conflicts of interest.

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